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Published in:
Journal of Applied Microbiology

DOI:
10.1111/j.1365-2672.2006.02911.x

Publication date:
2006

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Single cell analysis of gene expression patterns of competence development and initiation of sporulation in *Bacillus subtilis* grown on chemically defined media

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Keywords
Bacillus subtilis, biofilm, bistability, competence, differentiation, noise, sporulation, stochastic.

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2005/0962: received 25 August 2005, revised 28 September 2005 and accepted 28 September 2005

doi:10.1111/j.1365-2672.2006.02911.x

Abstract

Aim: Understanding the basis for the heterogeneous (or bistable) expression patterns of competence development and sporulation in *Bacillus subtilis*.
Methods and Results: Using flow cytometric analyses of various promoter-GFP fusions, we have determined the single-cell gene expression patterns of competence development and initiation of sporulation in a chemically defined medium (CDM) and in biofilms.
Conclusions: We show that competence development and initiation of sporulation in a CDM are still initiated in a bistable manner, as is the case in complex media, but are sequential in their timing. Furthermore, we provide experimental proof that competence and sporulation can develop under conditions that normally do not trigger these processes.
Significance and Impact of the Study: Some pathogens are able to develop natural competence, which is a serious medical problem with the increased acquired multi-drug resistance of these organisms. Another adaptive microbial response is spore formation. Because of their heat resistance and hydrophobicity, spores of a variety of species are of major concern for the food industry. Using the model organism *B. subtilis*, we show that competence development and sporulation are initiated in a bistable and sequential manner. We furthermore show that both processes may be noise-based, which has major implications for the control of unwanted differentiation processes in pathogenic and food-spoilage micro-organisms.

Introduction

Among the Gram-positive bacteria, many pathogens and food-spoilage species have been identified (for reviews see Jespersen and Jakobsen 1996; Jones *et al.* 1999; Ohlsen *et al.* 2004). Some pathogens are able to develop natural competence and, as a consequence of this adaptive strategy, show increased antibiotic resistance (Lorenz and Wackernagel 1994; Claverys *et al.* 2000). Resistance to a wide-range of antibiotics poses a serious epidemic threat for the community (McCormick 1998; Dobay *et al.* 2004; Walsh and Amyes 2004). Moreover, the occurrence of highly resistant spores at different stages in the production of food products, causes serious problems with regard to food preservation and safety (Brown 2000; Raso and Barbosa-Canovas 2003) and compromises medical hygiene as well.

Instead of trying to develop novel antibiotics to kill pathogenic or food-spoilage bacterium, one could try to identify compounds that inhibit the development of the unwanted differentiation process (*anti-differentics*). In the case of sporulation, for instance, a compound that would inhibit this process would render the species susceptible to normal methods of sterilisation. In the case of competence, *anti-differentics* could reduce the spread of drug-resistance. For this reason, more information is desired

about the molecular basis underlying these developmental processes. To study the origin of competence development and spore formation, we employ the Gram-positive model organism *Bacillus subtilis*. This rod-shaped bacterium is one of the best-studied microbial organism that exhibits cellular differentiation. Following exponential growth, *B. subtilis* is able to secrete several proteins (e.g. proteases, antimicrobials and pheromones) into its extracellular environment, develop competence for DNA uptake and form highly resistant endospores (for reviews see Errington 1993; Chen and Dubnau 2004; Tjalsma *et al.* 2004).

Competence in *B. subtilis* develops under specific nutritional conditions during late exponential and early stationary growth phase (Dubnau 1991). To initiate competence, the key regulator ComK needs to be expressed. This protein in turn activates expression of more than 100 genes, among which those required for DNA-uptake and recombination (Hamoen *et al.* 2002).

Spore formation in *B. subtilis* is a last resort adaptive response to starvation (Sonenshein 2000; Piggot and Losick 2002). It is controlled by a multi-component phosphorelay in which several stimuli can be integrated and cause phosphorylation (activation) of the key sporulation regulator, Spo0A (Burbulys *et al.* 1991). Activation of Spo0A affects the expression of more than 10% of all the genes in the genome of *B. subtilis* and can eventually lead to the formation of a highly heat resistant spore (Fawcett *et al.* 2000).

Competence and sporulation are subjected to multiple regulatory mechanisms and share many regulators in their gene-circuits (e.g. AbrB, SinR, DegU, CodY and Spo0A) (Msadek 1999; Hamoen *et al.* 2003). Certain conditions that stimulate competence development have a negative effect on sporulation and vice versa. However, other inputs stimulate or repress both gene-regulatory networks (Msadek 1999; Hamoen *et al.* 2003). Fascinatingly, when proper conditions are applied to a *B. subtilis* culture, only part of the population becomes competent or forms spores (Cahn and Fox 1968; Hadden and Nester 1968; Chung *et al.* 1994). Since, in both cases, there are clearly two distinguishable cell types, the systems were described as exhibiting bistability (Fujita *et al.* 2005; Maamar and Dubnau 2005; Smits *et al.* 2005b; Veening *et al.* 2005). We have previously shown that the observed bistability is the result of a non-linear auto-stimulation on *comK* and *spo0A*, respectively (Smits *et al.* 2005b; Veening *et al.* 2005). This was demonstrated using promoter-Green Fluorescent Protein (GFP) fusions combined with microscopic and flow cytometric analyses. However, single cell studies on competence development and initiation of sporulation have so far only been performed in complex, undefined media and never within the same (isogenic) strain. To determine whether competence and sporulation are processes that are mutually exclusive or simultaneously initiated, we make use of a chemically defined medium (CDM), in which both processes are initiated. This may resemble the conditions in industrially relevant processes, where chemically defined media are often used (e.g. Fang and Demain 1989; Vierheller *et al.* 1995; Huang *et al.* 2004). Therefore, we set out to perform single cell analyses on the expression patterns of both competence development and initiation of sporulation in chemically defined media. To further extend this, we composed a CDM in which both processes are initiated.

While previous studies were performed in liquid media, bacterial contaminations observed in industry and hospitals are often derived from dense biofilms. Therefore, we set out to determine the single cell gene expression patterns of competence and sporulation in a variety of biofilms.

The mechanism that decides, within an isogenic and homogeneous population, which cell actually reaches the threshold level necessary to activate auto-stimulation and become competent or to form a spore remains elusive. It was shown that stochastic fluctuations (or noise) of regulatory components within certain gene-regulatory pathways, can cause bistability (Hasty *et al.* 2000; Isaacs *et al.* 2003). In the establishment of this kind of bistability, noise in gene expression is supposed to play a key role. We quantitatively analysed the noise, for both competence and sporulation, in both liquid cultures and biofilms. We show that both competence development and initiation of sporulation show significant noise. This means that a small part of the population does not confine to the general rules of gene regulation and becomes competent or forms spores under conditions that normally should not trigger the initiation of these processes.

Materials and methods

Media and growth conditions

Chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck, or Baker Chemical Co. Liquid *B. subtilis* cultures were grown at 37°C and continuously shaken at 230 rpm, unless indicated otherwise. To study the development of competence and sporulation in colonies, cells were spread onto plates consisting of the indicated media type, supplemented with 1·5% agar and incubated overnight at 37°C. Biofilms with a liquid–air interface were obtained by inoculating *B. subtilis* directly from a frozen stock (−80°C) to 10 ml of CDM as indicated, and subsequent incubation at 37°C for 72 h without shaking.

The TY medium (tryptone/yeast extract) contained Bacto-Tryptone (1%), Bacto-yeast extract (0·5%) and NaCl (1%). Sporulation medium (SM) was prepared as described before (Schaeffer *et al.* 1965) and contained dehydrated nutrient broth (0·8%), NaOH (0·5 m mol l^{-1}), $MgSO_4$ (1 m mol l^{-1}), KCl (1 g l^{-1}), $Ca(NO_3)_2$ (1 m mol l^{-1}), $MnCl_2$ (0·01 m mol l^{-1}) and $FeSO_4$ (0·001 m mol l^{-1}). Minimal medium (MM) was prepared as described before (Leskela *et al.* 1996) and contained K_2HPO_4 (62 m mol l^{-1}), KH_2PO_4 (44 m mol l^{-1}), $(NH_4)_2SO_4$ (15 m mol l^{-1}), sodium citrate (6·5 m mol l^{-1}), $MgSO_4$ (0·8 m mol l^{-1}), 0·02% of casamino acids, glucose (27·8 m mol l^{-1}) and L-tryptophan (0·1 m mol l^{-1}). The pH was set to seven using a KOH solution. Chemically defined competence medium (CDCM) was prepared as described before (Smits *et al.* 2005a) and contained K_2HPO_4(62 m mol l^{-1}), KH_2PO_4(44 m mol l^{-1}), $MnCl_2$ (0·05 m mol l^{-1}), $(NH_4)_2SO_4$ (15 m mol l^{-1}), sodium citrate (6·5 m mol l^{-1}), $MgSO_4$ (0·8 m mol l^{-1}), glucose (27·8 m mol l^{-1}), L-glutamic acid (5·35 m mol l^{-1}) and L-tryptophan (0·1 m mol l^{-1}). The pH was set to seven using a KOH solution. Chemically defined sporulation medium (CDSM) was prepared as described before (Hageman *et al.* 1984) and contained MOPS (40 m mol l^{-1}, pH 7), KH_2PO_4 (4·0 m mol l^{-1}), $(NH_4)_2SO_4$ (9·5 m mol l^{-1}), L-lactate (5·0 m mol l^{-1}), L-glutamic acid (8·0 m mol l^{-1}), L-tryptophan (0·1 m mol l^{-1}), D-glucose (20 m mol l^{-1}) and 1 × MT mix. A 50 × MT mix was prepared as described before (Vasantha and Freese 1980) and contained $MgCl_2$(0·200 mol l^{-1}), $CaCl_2$ (70·0 m mol l^{-1}), $MnCI_2$ (5·00 m mol l^{-1}), $ZnCl_2$ (0·10 m mol l^{-1}), thiamin-hydrochloride (0·20 m mol l^{-1}), HCl (2·00 m mol l^{-1}) and $FeCl_3$ (0·50 m mol l^{-1}). To obtain a CDM in which both competence and sporulation are initiated, CDCM and CDSM were mixed in a 1 : 1 ratio (50/50). When appropriate, media for *B. subtilis* were supplemented with chloramphenicol (Cm; 5 μg ml^{-1}), spectinomycin (Sp; 100 μg ml^{-1}) or kanamycin (Km; 10 μg ml^{-1}).

Strains and plasmids

Table 1 shows the plasmids and bacterial strains used. All strains used in this study are isogenic derivatives of *B. subtilis* 168 (Kunst *et al.* 1997). *B. subtilis* was transformed as described before (Leskela *et al.* 1996).

Bacillus subtilis strain comG-gfp was obtained by a Campbell-type integration (single crossover) of plasmid pSGComGA (Smits *et al.* 2005b) into the chromosomal *comGA* promoter region of *B. subtilis* 168. Transformants were selected on TY agar plates containing Cm, after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown). *B. subtilis* strain IIA-gfp/*Δ*comK (P_{spoIIA}–*gfp*, *ΔcomK*) was obtained by transformation of strain IIA-gfp (Veening *et al.* 2005) with chromosomal DNA of strain BV2004 (Hamoen *et al.* 2002). Transformants were selected on TY agar plates containing Cm and Sp, after overnight incubation at 37°C. *B. subtilis* strain IIA-gfp/*Δ*spo0A (P_{spoIIA}–*gfp*, *Δspo0A*) was obtained by transformation of strain IIA-gfp (Veening *et al.* 2005) with chromosomal DNA of strain SWV215 (Xu and Strauch 1996). Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. *B. subtilis* strain G-gfp/*Δ*spo0A (P_{comG}–*gfp*, *Δspo0A*) was obtained by transformation of strain comG-gfp with chromosomal DNA of strain SWV215 (Xu and Strauch 1996). Transformants were selected on TY agar plates containing Cm and Km, after overnight incubation at 37°C. *B. subtilis* strain G-gfp/*Δ*comK (P_{comG}–*gfp*, *ΔcomK*) was obtained by transformation of strain comG-gfp with chromosomal DNA of strain BV2004 (Hamoen *et al.* 2002). Transformants were selected on TY agar plates containing Cm and Sp, after overnight incubation at 37°C.

Table 1 Bacterial strains and plasmids

Strains and plasmids	Genotype	Reference
B. subtilis		
168	*trpC2*	Kunst *et al.* (1997)
IIA-gfp	168, P_{spoIIA}–*gfp*, Cm^r	Veening *et al.* (2005)
IIA-gfp/*Δ*comK	168, P_{spoIIA}–*gfp*, Cm^r, *comK*::Sp, Sp^r	This study
IIA-gfp/*Δ*spo0A	168, P_{spoIIA}–*gfp*, Cm^r, *spo0A*::Km, Km^r	This study
comG-gfp	168, P_{comG}–*gfp*, Cm^r	This study
G-gfp/*Δ*spo0A	168, P_{comG}–*gfp*, Cm^r, *spo0A*::Km, Km^r	This study
G-gfp/*Δ*comK	168, P_{comG}–*gfp*, Cm^r, *comK*::Sp, Sp^r	This study
BV2004	*comK*::Sp, Sp^r	Hamoen *et al.* (2002)
SWV215	*Spo0A*::Km, Km^r	Xu and Strauch (1996)
Plasmids		
pSGComGA	*bla*, *cat*, P_{comG}–*gfp*	Smits *et al.* (2005b)

Flow cytometric analyses

Cells were 100 × diluted in 0·2 μ mol l^{-1} filtered MM and directly measured on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Mijdrecht, Netherlands) operating an argon laser (488 nm) essentially as described (Smits *et al.* 2005b; Veening *et al.* 2005). For each sample, at least 20 000 cells were analysed. To determine levels of 'noise' (see Table 4), at least 100 000 cells were analysed. Data containing the green fluorescent signals were collected by a FITC filter and the photomultiplier voltage was set between 700 and 800 V. Data was captured using EXPO32 software (Beckman Coulter) and further

analysed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). Figures were prepared for publication using WINMDI 2.8 and COREL GRAPHICS SUITE 11. To distinguish background fluorescence from GFP specific fluorescence, parental strain *B. subtilis* 168 was also analysed with each flow cytometric experiment.

To analyse biofilms, samples were first resuspended in 1 ml of MM and subsequently homogenized for 1 min using a mini bead-beater (Biospec Products, Bartlesville, OK, USA) before flow cytometric analysis. The gate of the forward and side scatter of the flow cytometer (which indicates the particle size) were set such that only single cells were counted for fluorescence analysis.

Competence and sporulation assays

Strain comG-gfp was grown in 3 ml of the chemically defined 50/50 medium and initiation of competence was determined by flow cytometric analysis at hourly intervals. Plasmid DNA (pWK-Sp; unpublished cloning vector containing a Sp marker between the flanking regions of the *amyE* gene of *B. subtilis*) was added to the culture when the presence of *comG-gfp* expressing cells first appeared. Incubation was continued for another 24 h, and the presence of phase-bright spores was verified by phase-contrast microscopy. To determine transformation frequencies, aliquots of the culture were diluted and plated onto TY agar plates without (viable count) or with (transformation count) Sp. Furthermore, to determine the total viable spore population and the transformed spores formed, samples were treated with a 1/10 volume of chloroform for 10 min (killing vegetative cells, while the chloroform-resistant spores survive). Chloroform treated samples were plated on TY (spore count) and on TY plates containing Sp (transformed spore count).

Results

Competence and sporulation are sequential processes

Competence development is initiated when a threshold level of ComK is reached and comK auto-activation is triggered (Maamar and Dubnau 2005; Smits *et al.* 2005b). ComK in turn activates the expression of the *comG* operon, which encodes factors required for the uptake of exogenous DNA from the environment (Chung and Dubnau 1998). Previously, it was shown that the promoter of the *comG* operon fused to the *gfp* gene is a good reporter to identify cells that have initiated competence development (Smits *et al.* 2005b). Since this reporter was so far only used in *B. subtilis* 8G5, a strain that poorly sporulates (Bron and Venema 1972), we transformed this construct to the completely sequenced and sporulating *B. subtilis* strain 168 (Kunst *et al.* 1997), resulting in strain comG-gfp (P_{comG}–*gfp*). To study initiation of sporulation at the single cell level, we made use of the previously published IIA-gfp strain (*B. subtilis* 168 P_{spoIIA}–*gfp*) (Veening *et al.* 2005). The *spoIIA* operon comprises *spoIIAA, spoIIAB* and *sigF*, a forespore-specific sigma factor essential for sporulation. The operon is activated at a high threshold level of activated Spo0A and was shown to be a good reporter for cells that initiate sporulation (Chung *et al.* 1994; Fujita *et al.* 2005; Veening *et al.* 2005).

Previous single cell analysis using these two reporters were performed in complex undefined media that are optimized to either study competence or sporulation (Smits *et al.* 2005b; Veening *et al.* 2005). To examine whether one of these media or a combination of both is suited for the analysis of both competence and sporulation and to examine whether the processes are mutually exclusive or happen simultaneously, flow cytometric analyses of strains comG-gfp and IIA-gfp were performed in both MM and SM (Fig. 1). As shown in Fig. 1a, competence is not initiated in SM (left panel) and sporulation not in MM (right panel). Expression histograms of strains comG-gfp and IIA-gfp grown in dedicated media were published before (Smits *et al.* 2005b; Veening *et al.* 2005). Furthermore, when the two media are mixed in a range of ratios, either competence or sporulation development is poor. Interestingly, there is a low level of expression of *spoIIA-gfp* when cells were grown in MM (Fig. 1a, right panel). This is most likely caused by repression (or activation) of low-threshold Spo0A genes (see Discussion).

To study both processes simultaneously and get a better idea of the timing and possible co-occurrence of the developmental pathways, we examined the expression pattern of strains comG-gfp and IIA-gfp in CDCM (Smits *et al.* 2005a), CDSM (Hageman *et al.* 1984) and a mix of both media in a 1 : 1 ratio (50/50). As shown in Fig. 1b, both competence (left panel) and sporulation (right panel) are initiated in a bistable manner in the 50/50 chemically defined media. With increasing concentrations of CDSM, more cells initiate competence development as well as sporulation, although the timing of sporulation differs slightly between the media (data not shown). For further analysis we chose to continue with the 50/50 medium, since under these conditions both processes seem to be initiated discretely. To better visualize the timing of both processes, an overlay of the expression patterns of strains comG-gfp and IIA-gfp was made in which samples were taken of both strains at identical points in their growth phase (Fig. 1c). For this image, flow cytometric histograms were taken from cultures independent of the

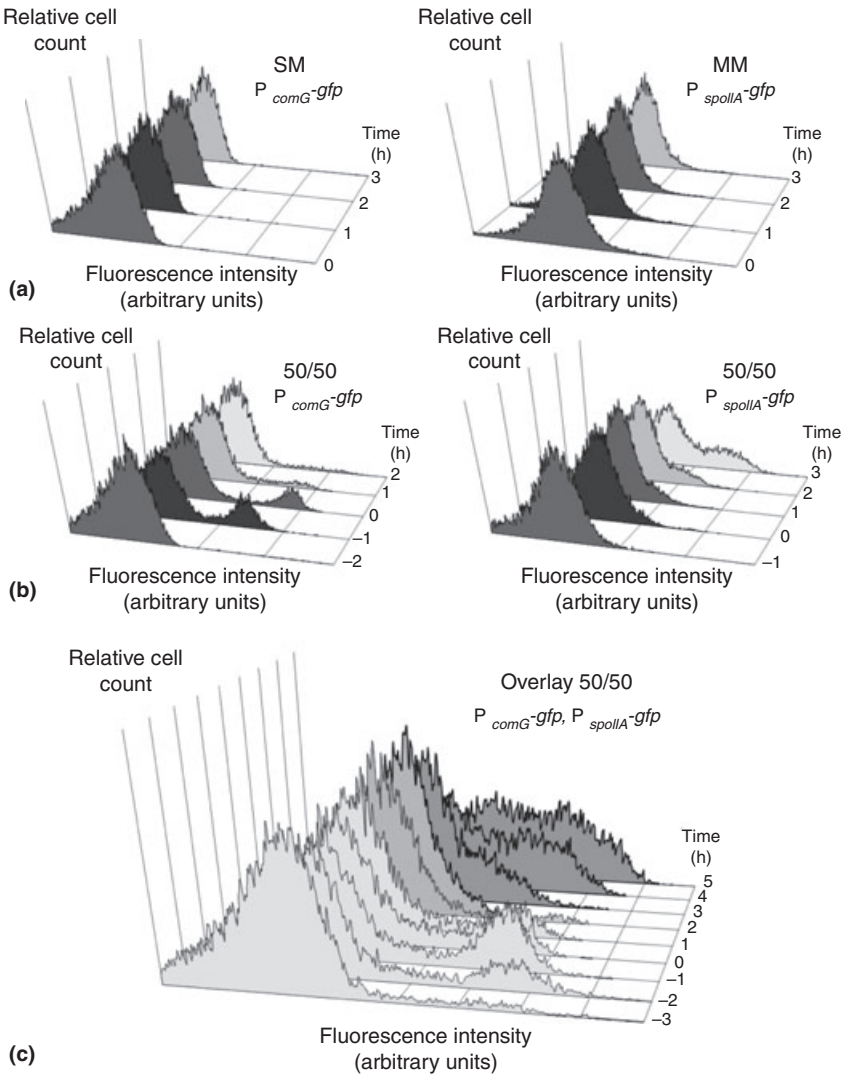

Figure 1 Single cell analysis of P_{comG}–*gfp* and P_{spollA}–*gfp* in liquid media. Time is given in hours relative to the transition point between the exponential and stationary growth phase (T0). (a) Strains comG-gfp (left panel) and IIA-gfp (right panel) were grown in liquid SM and MM, respectively. (b) Strains comG-gfp (left panel) and IIA-gfp (right panel) were grown in liquid 50/50 (chemical defined) medium. (c) An overlay of single cell expression patterns of strains comG-gfp (light gray histograms) and IIA-gfp (dark gray histograms) in 50/50 medium. Histograms of $t = 2$ samples from both strains are indicated with intermediate gray shading, since this point shows the last measured fluorescence from the competence specific GFP-reporter strain and the first analysis from the strain harboring the sporulation specific GFP reporter.

cultures used in Fig. 1b. As shown in this figure, competence development and sporulation are sequentially initiated; first a subpopulation of cells becomes competent and later a subpopulation initiates sporulation. Interestingly, competence is already initiated during late-logarithmic growth, in contrast to growth in complex medium where expression of *comG* is generally not observed until 2 h after entry into stationary growth phase (Smits *et al.* 2005b). Furthermore, sporulation is initiated from approximately 2 h after entry into the stationary growth phase, while in SM, this occurs already at transition point (Veening *et al.* 2005). Overall, these results indicate that in the 50/50 CDM, both developmental processes occur but appear to differ in timing. Furthermore, competence bistability is clearly reversible, whereas sporulation bistability seems to be a unidirectional process under the conditions tested.

Competent cells can enter sporulation

To answer whether or not the two developmental pathways are mutually exclusive (can competent cells sporulate?), we added an integrative plasmid DNA containing an antibiotic resistance marker to the culture in the timeframe where fluorescence was observed for the comG-gfp reporter strain. Incubation was continued until 24 h after the start of growth and cells were plated on selective and non-selective plates (to determine transformation frequency and viable count respectively). Furthermore, at this time-point samples were treated with chloroform to kill vegetative cells and the remaining chloroform resistant spores were plated on selective and non-selective plates (to determine the transformed, and non-transformed spore counts, respectively). As shown in Table 2, some spores were found to be resistant to spectinomycin.

Table 2 Competent cells are able to sporulate

Fraction	Percentage†
Viable count	100
Spore count	12
Transformed cells	60×10^{-7}
Transformed spores	14×10^{-7}

†To determine competence and sporulation efficiencies, cells were plated onto TY agar plates as described in the Materials and Methods section. After overnight incubation at 37°C, colony-forming units were counted and percentages were determined by normalizing on basis of the viable count.

This establishes that competent cells are not limited to this developmental pathway, but can enter the sporulation pathway at a later stage. Since the optical density of a culture in stationary phase does not increase further, but rather decreases, and competent cells are arrested in growth (Haijema *et al.* 2001), it is highly unlikely that cells which have been competent at the end of the logarithmic growth phase divide again and subsequently sporulate. More likely, cells first become competent, and subsequently have the ability to sporulate. However, using the *comG-gfp* reporter we show that competence is already initiated during late exponential growth (Fig. 1). This could mean that cells that become competent during logarithmic growth, could have some time to resume division and/or 'reset' and later initiate sporulation. Whether such a 'resetting' is required to initiate sporulation remains to be investigated. Time-lapse and/or double-labeled fluorescent microscopy could serve to address this hypothesis, and is currently under investigation in our laboratory.

Effects of a *comK* mutation on sporulation bistability

ComK is the master regulator for competence development. The protein activates more than 100 genes, amongst which those necessary for the uptake of DNA from the environment and integration into the genome (Hamoen *et al.* 2002). To date, no effects of ComK on sporulation have been reported. However, as mentioned before, these studies were mainly performed in media designed for efficient competence or sporulation and mutational effects were only studied on the level of population averages. The fact that in 50/50 medium competence development and initiation of sporulation are sequentially initiated, prompts the question whether competent cells actively compete with the initiation of sporulation in this medium. To test this hypothesis, we introduced a *comK* mutation in our sporulation reporter strain. The resulting strain, IIA-gfp/comK (P_{spoIIA}–*gfp*, *ΔcomK*), was grown in 50/50 medium and cells were analysed by flow cytometry. As shown in Table 3, a mutation in *comK* has no significant effect on the final bistable sporulation outcome. However, sporulation seems to initiate somewhat earlier in the *comK* mutant. This might be caused by the slight increase in growth rate that is observed for the *comK* mutant in the 50/50 CDM (data not shown). We are currently looking into the reasons of this increase.

Table 3 Competence does not significantly affect sporulation bistability

Time†	Wildtype	*ΔcomK*
Percentage of cells in the high *spoIIA* state¶		
1	0·1 ± 0·0	0·1 ± 0·0
2	2·5 ± 0·4	5·2 ± 0·7
3	22·6 ± 1·4	25·8 ± 1·1
4	39·6 ± 1·8	40·3 ± 2·2

¶Cells with a fluorescence level higher than channel 650 were considered to be in the high *spoIIA* state, as described before (Veening *et al.* 2005). An average of the percentage of highly fluorescent cells (related to the total cell count) of four independent experiments are shown; ± indicates the SE in these data.
†Time is given in hours relative to the transition between exponential and stationary growth phase (T0).

Competence and sporulation in biofilms

Above, it was demonstrated that in a CDM, competence and sporulation are still initiated in a bistable manner. The single cell analyses of both developmental processes have so far been limited to those performed in liquid media (Chung *et al.* 1994; Maamar and Dubnau 2005; Smits *et al.* 2005b; Veening *et al.* 2005). In practice, bacterial infections and contaminations are often observed in the form of dense biofilm-like structures (such as colonies on solid surfaces and pellicles on liquids). Under these circumstances, the expression patterns may be significantly different from those observed during growth under laboratory circumstances in which cells are homogeneously shaken and quorum sensors are more likely to be distributed equally throughout the culture. In biofilms however, quorum sensing plays a pivotal role in the initiation of adaptive responses such as competence and sporulation (see for instance Branda *et al.* 2001; Stanley *et al.* 2003; Ren *et al.* 2004). Therefore, we were interested to see the flow cytometric profiles of the reporter strains under these conditions. Strains comG-gfp (P_{comG}–*gfp*) and IIA-gfp (P_{spoIIA}–*gfp*) were inoculated on solid agar plates containing CDCM, 50/50 and CDSM and high-cell density multicellular structures in the form of colonies were allowed to form overnight at 37°C. The resulting colonies were homogenized and analysed by flow cytometry as described in the materials and methods. As shown

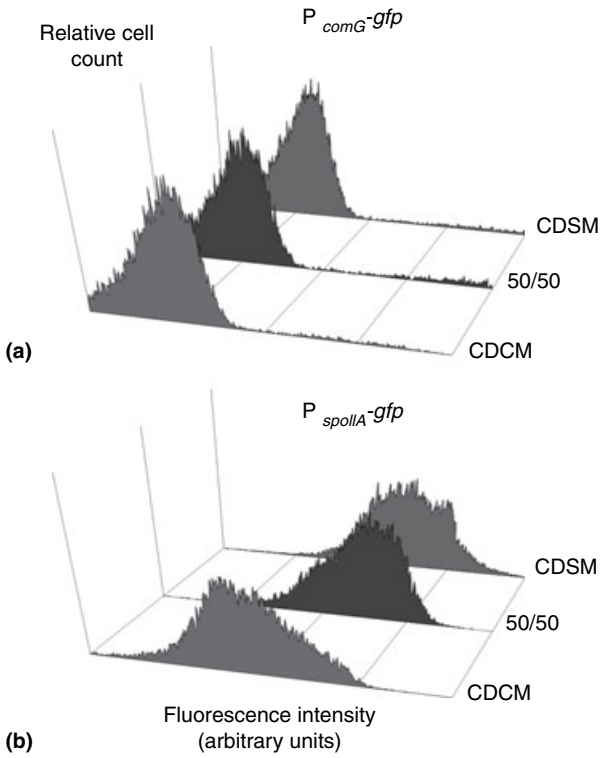

Figure 2 Competence and sporulation in colonies from solid media. Samples were prepared for flow cytometric analysis as described in the Materials and Methods section. Strains were allowed to form a colony on solid chemically defined media (indicated on the *Z*-axis) after 24 h of incubation at 37°C. (a) Strain comG-gfp (P_{comG}–*gfp*). (b) Strain IIA-gfp (P_{spollA}–*gfp*).

in Fig. 2a, in contrast to growth in liquid media, competence is poorly initiated when grown on solid chemically defined media (on solid MM competence is well initiated; see Table 4). Sporulation however, is still well initiated when grown on solid chemically defined media (Fig. 2b). With increasing concentrations of CDSM, more cells produce GFP expressed from the *spoIIA* promoter. However, the pattern is clearly less bistable when compared to initiation of sporulation in liquid chemically defined media. This suggests that sporulation is stronger induced in dense biofilms compared to growth in liquid shaken cultures under the conditions tested.

To determine the competence and sporulation expression patterns in liquid–air pellicle-like biofilms (as described by Branda *et al.* 2001), a standing 50 ml Greiner tube was filled with 10 ml of CDCM, 50/50 and CDSM, respectively, and strains comG-gfp and IIA-gfp were allowed to growth in these tubes for 72 h at 37°C without shaking. Cells were extracted from both the pellicle (liquid–air interface) and the liquid-phase (planktonic cells) and homogenized and analysed by flow cytometry as described in the Materials and Methods. As shown in

Table 4 Noise of competence and sporulation

	Biofilms		Liquid cultures	
Strain	MM†	SM‡	MM	SM
Percentage of highly fluorescent cells¶				
168	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00	0·00 ± 0·00
IIA	0·70 ± 0·18	21·06 ± 0·90	0·06 ± 0·00	91·30 ± 0·74
comG	13·02 ± 0·48	0·07 ± 0·01	4·58 ± 0·07	0·03 ± 0·00

¶Cells with a fluorescence level higher than channel 650 were considered highly fluorescent. An average of the percentage of highly fluorescent cells (related to the total cell count in which at least 10^5 cells were counted) of four independent experiments are shown; ± indicates the SE in these data.

†MM is minimal medium, which is commonly used to induce competence development (Leskela *et al.* 1996).

‡SM is sporulation medium, which is generally used to induce sporulation (Schaeffer *et al.* 1965).

Fig. 3 (left panels), competence development is poorly initiated in both the pellicle and standing liquid culture in all chemically defined media tested. While sporulation (Fig. 3, right panels) is poorly initiated in CDCM (Fig. 3a) and 50/50 (Fig. 3b) media, it is efficiently initi-

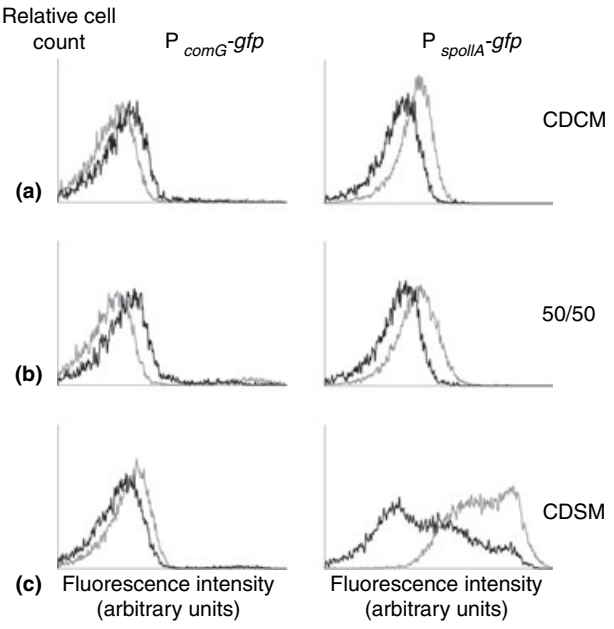

Figure 3 Gene expression patterns in biofilms. Samples were prepared for flow cytometric analysis as described in the materials and methods section. Strains were grown for 72 h at 37°C, without shaking. Black lined-histograms indicate gene expression profiles of cells extracted from the liquid-phase of the growth medium. Gray lined-histograms indicate gene expression patterns of cells extracted from the pellicle formed on top of the liquid medium. Strains comG-gfp (left panel) and IIA-gfp (right panel) grown in CDCM (a), 50/50 (b) and CDSM (c).

ated in CDSM (Fig. 3c). In all cases however, sporulation is more efficiently initiated in the pellicle.

Noise under non-stimulatory conditions

Recently, it was demonstrated that the bistability in gene expression that is observed in complex liquid media, requires the presence of a positive feedback loop involving the key-regulators of competence and sporulation (Maamar and Dubnau 2005; Smits *et al.* 2005b; Veening *et al.* 2005). This type of bistability is thought to originate from noise in the expression of the key-regulators. However, limited experimental studies have been performed to quantify this. Maamar and Dubnau (2005) postulated a role for noise in competence development based on the occasional observation of cells expressing the reporter under atypical conditions. Dawes and Thornley (1970) studied sporulation in *B. subtilis* using continuous chemostat cultures. They observed that even at very high-dilution rates, conditions that should not trigger sporulation, some cells sporulate. We propose that this may well be contributed to noise within the sporulation network. Motivated by these previous findings, we were interested in extending this work and quantitatively analyse the noise, if present, in both competence and sporulation in both liquid cultures and biofilms. We set out to quantify noise in our system, by looking at cells that initiate sporulation and competence under non-stimulatory conditions. To this end, we adapted the conditions for flow cytometric measurements, to count large numbers of cells (at least 100 000), and to filter out false negative signals by rigorous gating, which removes particles with a different size than a single cell from the analysis and only counts cells with a very high fluorescence level (*noise-measurement*). This strategy eliminates background signals and false-positives, but may also reduce the amount of true positives, and thus effectively gives an under-representation of the fluorescent signals. To obtain significant 'noise' data, it is essential to measure many single cells within a population. The use of flow cytometry in combination with promoter-GFP fusions as described in this work greatly facilitates such studies. Strains comG-gfp and IIA-gfp were grown on solid and liquid MM and SM respectively. To allow single cell analyses, colonies were homogenized and analysed by flow cytometry as described in the materials and methods. Cells from liquid cultures were immediately analysed on a flow cytometer. For each culture condition, four individual colonies or cultures were measured. As mentioned in the first paragraph of this study, no clear development of competence was observed on complex SM (Fig. 1a), nor sporulation on a complex medium dedicated to induce competence (Fig. 1b) using standard flow cytometric settings. However, using *noise-measurements*, it becomes clear that under all conditions tested, there are some cells that demonstrate development of competence or sporulation, despite the lack of triggers for these processes (Table 4). Importantly, a negative control (a strain without fluorescent reporter) gives no fluorescent signals at all under the conditions tested using *noise-measurements*. Furthermore, it seems that the sporulation pathway is noisier than the competence pathway. Interestingly, both developmental processes show a higher degree of noise in dense-biofilms compared to liquid cultures. Overall, by quantifying the atypical initiation of the developmental pathways for the first time, these observations strengthen the hypothesis that noise contributes to the establishment of heterogeneity for competence and sporulation. In addition, it suggests that the levels of noise differ between different cellular processes.

Discussion

Competence for genetic transformation and sporulation form a major problem for food and medical industry because they render bacteria insusceptible to conventional methods of sterilization and treatment. To effectively counter these bacterial adaptive strategies, one approach would be to specifically inhibit the unwanted developmental process. For this reason, detailed knowledge is required about the molecular mechanisms governing the development of competence and sporulation. Most studies so far have been performed under conditions in shaken batch cultures in complex media. These conditions may not reflect industrial or medical circumstances.

In literature, some investigations of competence and sporulation in chemically defined media are available. However, all the studies evaluate gene expression patterns at the population level instead of looking at single cell gene expression patterns (Hageman *et al.* 1984; Murayama *et al.* 2004). Here, we present the first single cell analysis of competence development and the initiation of sporulation in the same genetic background, in a CDM, and under biofilm-promoting conditions.

Our results demonstrate that also in chemically defined liquid media both competence and sporulation show a bistable distribution in gene expression (Fig. 1). Although the ratio between high- and low-expressing cells seems to vary depending on culturing conditions, bistability is independent of media.

As shown in Fig. 1a right panel, the expression of *spoIIA-gfp* is higher than the background fluorescence and all cells are in this so-called low state (Veening *et al.* 2005). Recently, it was found that, for instance *abrB*, is repressed by Spo0A at a low threshold level (Fujita *et al.* 2005). AbrB represses the gene encoding *sigH*, a gene encoding

an alternative sigma factor (Weir *et al.* 1991). Since the *spoIIA* operon is expressed from a sigma-H dependent promoter (Wu *et al.* 1991), the low level of expression observed in the right panel of Fig. 1b might be caused by the relief of AbrB-repression on the *sigH* promoter. For a high activation of *spoIIA* however, high levels of Spo0A are required (Fujita *et al.* 2005; Veening *et al.* 2005). This observation might also explain why competence development is stimulated in MM, since it was shown that to initiate competence, Spo0A needs to downregulate *abrB* and does this already at a low threshold level (Hahn *et al.* 1995; Fujita *et al.* 2005). Interestingly, not only the ratio between both populations seems to vary depending on the media, also the timing of the processes seems to differ between complex media and chemically defined media. Competence, for instance, is normally initiated in stationary growth phase in complex medium, whereas in the CDM, *comG* was already expressed during the late exponential growth phase (Fig. 1b, c). This difference could be due to the fact that in the CDM, cultures reach a much higher optical density than in complex competence medium. Since a key step in the development of competence is regulated through quorum sensing, the threshold for the quorum sensing response may be reached earlier. Consistent with this hypothesis, it was reported that overproduction of a downstream component of the quorum sensing event, ComS, leads to precocious and increased competence development (Maamar and Dubnau 2005). Next, it was found that competence and sporulation are subsequent processes (Fig. 1). The fact that amongst the total population of spores, it was possible to identify those transformed earlier in growth, indicates that the two developmental pathways are not mutually exclusive (Table 2). Competence bistability can therefore be classified as a reversible bistable process that does not exclude cells from entering the sporulation pathway. Through the introduction of the *comK* mutation in the IIA-gfp strain, it was established that abolishment of competence does not significantly affect the final bistable sporulation outcome (Table 3). This indicates that there is no direct relation between *comK* expression and the bistable expression pattern of sporulation genes. Taken together, these results form a strong argument to view both bistable processes as independently regulated under the conditions tested. The origin of this strong independency in regulation remains an intriguing question, since both processes respond to similar triggers (Dubnau 1991; Hoch 1991). In addition, the key-regulator of sporulation, Spo0A, is also essential for competence development. This was verified by single cell analysis of our reporter strains in which a *spo0A* mutation was introduced (comG-gfp/spo0A and IIA/spo0A). For both strains, the bistable expression patterns were abolished, and no fluorescence was observed (data not shown). It is important to realize that the fact that no bias is observed in sporulation of cells that have or have not been competent, does not exclude the existence of a regulatory mechanism that temporally separates the two developmental pathways.

A bistable expression pattern was also observed under biofilm-promoting conditions. Interestingly, development of spores seems to occur preferentially at the liquid air interface of pellicle-like biofilms on liquid. These results are consistent with the results of Branda and co-workers, in which it was shown that sporulation is preferably initiated within aerial structures of a biofilm (Branda *et al.* 2001). Formation of biofilms was recently reported to involve a master regulator, SinR (Kearns *et al.* 2005). Interestingly, SinR was initially identified as a sporulation inhibitor (Bai *et al.* 1993; Mandic-Mulec *et al.* 1995), indicating the close relationship between both processes. Competence in biofilms in CDM seems to be poorly developed (Figs 2 and 3), while in colonies grown on solid MM agar, competence is well initiated (Table 4). This difference might be attributed to the fact that for the flow cytometry analyses of colonies from CDM, cells were harvested after prolonged incubation, up to 72 h and for the noise measurements after 24 h. Since competence is a reversible process, cells may have reverted to a non-expressing state or have lysed. Interestingly, the cells that did express *comG*, showed very high levels of expression, and little or no intermediate levels of expression (Figs 2 and 3). Conceivably, in biofilms, micro-environments differ significantly between areas, and lead to strong induction of a specific developmental pathway.

Finally, the quantitative analysis of the expression of competence and sporulation reporters under atypical conditions indeed seems to suggest a role for noise in both processes (Table 4). Although unlikely, it cannot be excluded that some of the observed fluorescent cells demonstrate expression due to a secondary mutation. This could be tested by sorting out the 'noisy' cells and perform competence and/or sporulation efficiency assays. However, the results are in excellent agreement with previous reports that suggest that noise in gene expression is key to both competence (Maamar and Dubnau 2005) and sporulation, and that the decision to enter either of these pathways is stochastic (Maughan and Nicholson 2004). From an industrial or medical point of view, noise in gene expression has major implications. From our study, it becomes apparent that, although the number of expressing cells can significantly be reduced using specially developed media, there are always certain cells that behave atypically, due to the noisy character of the developmental routes. On a laboratory scale, these may be negligible, but in industrial fermentations or food-industrial

processes for instance, the occurrence of highly resistant spores in the system can have big consequences. Also, increased resistance to a wide-range of antibiotics, which can be linked to genetic transformation (Dobay *et al.* 2004), is more and more becoming a serious epidemic threat for the community. Therefore, future research must be aimed at localizing the noisiest regulatory components in developmental pathways and neutralizing these targets.

Acknowledgements

LWH was supported by a BBSRC Grant awarded to J. Errington. WKS and JWV were supported by Grant 811.35.002 and ABC-5587 from the Netherlands Organization of Scientific Research, Earth- and Lifesciences (NWO-ALW) and Technology Foundation (NWO-STW), respectively. JWV and WKS contributed equally to this study.

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